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ORIGINAL PAPER

Precursor-directed biosynthesis of 6-deoxyerythronolide B analogues is improved by removal of the initial catalytic sites of the polyketide synthase

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Abstract Precursor-directed biosynthesis has been shown to be a powerful tool for the production of polyketide analogues that would be difficult or cost prohibitive to produce from medicinal chemistry efforts alone. It has been most extensively demonstrated using a KS1 null mutation ($KS1^0$) to block the first round of condensation in the biosynthesis of the erythromycin polyketide synthase (DEBS) for the production of analogues of its aglycone, 6-deoxyerythronolide B (6-dEB). Here we show that removing the DEBS loading domain and first module (mod1Δ), rather than using the $KS1^0$ system, can lead to an increase in the utilization of some chemical precursors and production of 6-dEB analogues (R-6dEB) in both *Streptomyces coelicolor* and *Saccharopolyspora erythraea*. While the difference in utilization of the precursor was diketide specific, in strains fed (2*R**, 3*S**)-5-fluoro-3-hydroxy-2-methylpentanoate *N*-propionylcysteamine thioester, twofold increases in both utilization of the diketide and 15-fluoro-6dEB (15F-6dEB) production were observed in *S. coelicolor*, and *S. erythraea* exhibited a tenfold increase in production of 15-fluoro-erythromycin when utilizing the mod1Δ rather than the $KS1^0$ system.

Keywords Polyketide · Erythromycin · Genetic engineering

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Introduction

Polyketides are a large and diverse group of natural products that have a wide variety of medically important biological activities such as antibiotic, antifungal, and anti-cancer. The modular polyketide synthases (PKSs) that produce these complex molecules use simple building blocks in a mechanism similar to fatty acid synthesis [8, 20, 25]. The great structural diversity among polyketides comes from the choice of extender unit utilized and the different extents of reduction at each condensation step, as well as the possible wide array of tailoring enzymes available for post-polyketide modification [19, 20, 25, 26]. It has been shown that the catalytic domains of PKSs can be inactivated to allow for bypass of the early steps in polyketide biosynthesis and incorporation of chemical precursors to generate novel polyketide analogues [11, 14].

The erythromycin aglycone precursor 6-deoxyerythronolide B (6-dEB) is formed by the processive condensation (and reduction) of one propyl-CoA primer and six methylmalonyl-CoA extender units catalyzed by 6-dEB synthase (DEBS), which is composed of three distinct proteins, DEBS1, DEBS2, and DEBS3 [12]. Although normally found in the erythromycin-producing host *Saccharopolyspora erythraea*, the DEBS genes have been expressed in a number of heterologous hosts resulting in the production of 6-dEB [12, 16, 21, 22, 24]. Precursor-directed biosynthesis of 6-dEB was initially performed in *Streptomyces coelicolor* utilizing a null mutation in the ketosynthase (KS) domain of the first module located in DEBS1 [5, 11]. This mutation ($KS1^0$) blocks the biosynthesis of the natural product as the PKS is unable to catalyze the first round of condensation. However, production

can be restored by addition of a synthetic thioester diketide that mimics the structure of the intermediate generated from the first condensation cycle. The diketide enters the cell and can form the required thioester linkage with the second module of the PKS [3, 11, 27]. By varying the structure of the diketide employed, this process has opened up an avenue for production of a variety of novel analogues. The yield of product is reduced, however, possibly due to decreased efficiency of substrate utilization and degradation of the synthetic precursor [7, 11, 17, 18]. A truncated version of DEBS 1, consisting only of the second module along with the DEBS thioesterase (TE) was shown to exhibit an increase in diketide utilization during the production of triketide lactones (R-TKLs) when compared with the DEBS1-TE construct that carried $KS1^0$ mutation in *S. coelicolor* [23].

We were interested to determine whether the truncated version of DEBS 1 would lead to similar increases in utilization of a number of diketide precursors in systems employing the entire set of DEBS genes, both in *S. erythraea* and *S. coelicolor*. We found that the truncated version (mod1Δ) consistently showed increased diketide utilization over its $KS1^0$ counterpart in both hosts and with a variety of precursors.

Materials and methods

Racemic diketide thioesters

Enantioselective synthesis of (2*S*, 3*R*)-3-hydroxy-2-methyl-4-pentenoate *N*-acetylcysteamine thioester (vinyl-SNAC) was preformed as previously described [2]. Diastereoselective synthesis of racemic (2*R**, 3*S**)-4-chloro-3-hydroxy-2-methylbutanoate *N*-propionylcysteamine thioester (ClMe-SNPC) (2*R**, 3*S**)-3-hydroxy-2-methylhexanoate *N*-propionylcysteamine thioester (Pr-SNPC), and (2*R**, 3*S**)-5-fluoro-3-hydroxy-2-methylpentanoate *N*-propionylcysteamine thioester (Fet-SNPC) were preformed as previously outlined [1].

Bacteria and plasmids

Bacteria and plasmids used in this study are shown in Table 1 *S. coelicolor* PF6–10 was created through a strain improvement program from the parent strain *S. coelicolor* CH999/1126*, which contains the full DEBS genes on a co-integrated, high copy SCP2 vector. This strain was cured of its plasmid to create the optimized "clean" host *S. coelicolor* K146-151B [4]. To create a plasmid for the expression of the truncated DEBS under the actinorhodin promoter, *actIp*, in a SCP2 vector, the DEBS expression plasmid pKOS146-103A [4] was cut with SpeI and HindIII to obtain a *ca.* 34.1 kb fragment and with HindIII and NdeI to generate a *ca.* 3.7 kb fragment. The plasmid pKOS214-119 [23], which contains DEBS module 2 with a N-terminal DEBS module 5 linker, was digested with NdeI and SpeI generating a *ca.* 4.3 kb fragment. These three fragments were ligated to give the expression plasmid pKOS279-7A. The plasmids pKOS146-145 (pBOOST [9]) for increasing SCP2 plasmid copy number and pJRJ2 [11], which is a DEBS ($KS1^0$) expression vector similar to pKOS279-7A, have been described previously. The suicide vector for homologous recombination in *S. erythraea* to produce the desired truncated DEBS1 contained flanking regions from the *eryAIp* and DEBS module 2. The plasmid pKOS97-49B [10] was digested with EcoRI and a blunt end was created by a 5′ overhang fill using DNA Polymerase I. The DNA was then digested with NdeI to produce a *ca.* 4 kb fragment. That fragment was ligated with a *ca.* 1.3 kb fragment from digesting pKOS279-160B with PmlI and NdeI to create pKOS460-102.

Strain construction

Plasmid pKOS146-145 (which generates an increased plasmid copy number after co-integration with SCP2* plasmids) and pKOS279-7A were introduced into *S. coelicolor* CH999 and K146-151B by protoplast transformation [15] employing selection for apramycin and thiostrepton resistance. The strains *S. coelicolor* K279-7A* and *S. coelicolor* K460-2 were selected from among the transformants. The strains *S. coelicolor* JRJ2* and B9, which contain the DEBS PKS with a KS1 null mutation and a boosted plasmid copy number, were made previously [4].

To create a deletion of the loading domain and module 1 of DEBS in *S. erythraea*, pKOS460-102 was introduced into the strain by conjugation from *Escherichia coli* ET12567/pUB307 according to standard protocols [6]. Replacement of the segment of DEBS in the *S. erythraea* chromosome by double reciprocal recombination with the incoming plasmid was confirmed by PCR.

Culture conditions

Isolation of individual colonies employed growth on R5 agar plates [13]. Spores were obtained by plating on MS [13] or M1 agar [24] supplemented with 50 mg/L apramycin and/or 50 mg/L thiostrepton, as required. For most *S. coelicolor* fermentations, SCVM6-1 was

Table 1 Bacteria and plasmids

Strain/plasmid designation	Description	Reference
Streptomyces coelicolor JRJ2*	Isolate of *Streptomyces coelicolor* CH999 with DEBS/KS1^0 on plasmid pJRJ2 co-integrated with plasmid pKOS146-145	[18]
Streptomyces coelicolor K279-7A*	Isolate of *Streptomyces coelicolor* CH999 with DEBS/mod1Δ on plasmid pKOS279-7A co-integrated with pKOS146-145	This work
Streptomyces coelicolor K146-151B	Improved host derived from a strain improvement program and cured of its plasmids	[4]
Streptomyces coelicolor B9	Isolate of *Streptomyces coelicolor* K146-151B with DEBS/KS1^0 on pJRJ2 co-integrated with pKOS146-145	[4]
Streptomyces coelicolor K460-2	Isolate of *Streptomyces coelicolor* K146-151B with DEBS/mod1Δ on plasmid pKOS279-7A co-integrated with pKOS146-145	This work
Saccharopolyspora erythraea K41-135	Erythromycin over-producing strain	[24]
Saccharopolyspora erythraea K39-14	Isolate of *Saccharopolyspora erythraea* K41-135 containing a mutation in the chromosome to create KS1^0 in DEBS module 1	[7]
Saccharopolyspora erythraea K460-126A	Isolate of *Saccharopolyspora erythraea* K39-14 in which the loading domain and first module of DEBS have been removed	This work
pJRJ2	*Streptomyces* SCP2* based plasmid containing DEBS1with KS1^0, DEBS2, and DEBS3 under actIp	[11]
pKOS279-7A	*Streptomyces* SCP2* containing DEBS1 with mod1Δ, DEBS2, and DEBS3 under actIp	This work
pKOS146-145 (pBOOST)	SCP2 based plasmid that increases plasmid copy number after co-integration with a SCP2* plasmid in *Streptomyces*	[9]
pKOS460-102	Suicide plasmid for double reciprocal recombination in *Saccharopolyspora erythraea* to generate DEBS mod1Δ system	This work

used as the seed medium and SCFM6-2 for shake flask fermentations [4]. For production of erythromycin in *S. erythraea*, F1-S medium, which contains 3.5% (w/v) starch, 3.2% dextrin, 0.7% NaCl, 1.2% corn steep liquor (50%), 0.2% $(NH4)_2SO_4$, and 0.8% $CaCO_3$, was used. For protein analysis, *S. coelicolor* and *S. erythraea* were grown in TSB seed medium and R5 liquid medium for shake flask fermentation [15]. Seed culture medium was supplemented with 50 mg/L apramycin and/or 20 mg/L thiostrepton, as required. Strains were maintained as spores in 30% glycerol and frozen cell banks were made taking an exponentially growing culture in seed medium, adding glycerol (30% v/v final), and freezing in 1 mL samples at − 80 °C.

Seed cultures were established using one cell bank vial to inoculate 50 mL of SCVM6-1 or TSB and cultivated at 30 °C for 3 days shaking at 200 rpm. For polyketide production, duplicate flasks containing 35 mL of SCFM6-2, R5, or F1-S were inoculated with 1.75 mL (5% v/v) of seed culture. These cultures were incubated at 30 °C and 200 rpm. After growth for 40–48 h, the diketide precursor (400 g/L stock in DMSO) was added to the production cultures at final concentrations of 1–4 g/L, as specified and the cultures were incubated for a further 6 days. After the initial bolus of diketide, some cultures were given an additional feed of 0.5 g/L/day on days 3–7. Samples were withdrawn as 500 µL aliquots as indicated and stored at −20 °C. At each sampling, flasks were weighed to follow water loss.

Analyses

For analysis of the diketide, Fet-SNPC, and 6dEB analogues, culture broth was diluted 1:1 with methanol and mixed for at least 1 h. Samples were then centrifuged at 12,000×*g* for 10 min and the supernatant was analyzed by HPLC. Quantitation was performed using a Hewlett-Packard 1090 HPLC equipped with a diode array detector (DAD) and an Alltech 500 evaporative light scattering detector (ELSD). Supernatant was diluted as necessary, and 4 µL was injected onto a guard column (4.6 × 10 mm^2 Inertsil ODS3-5 µm, Varian Analytical Instruments, Walnut Creek, CA, USA) and main column (4.6 × 50 mm^2 Inertsil ODS3-5 µm, Varian Analytical Instruments) held at 50 °C. The assay method consisted of an extraction with 100% water for 2 min, a 6 min gradient separation starting from 100% water and ending at 100% acetonitrile, a 1 min elution at 100% acetonitrile, and followed by equilibration at 100% water for 3 min (1 mL/min flow rate). The 6-dEB analogues (R-6dEB) were detected and quantitated by ELSD. Fet-SNPC eluted at 6.2 min and was detected by UV absorbance at 250 nm. Quantitation of R-6dEB was performed using a standard curve [100–500 mg/L 15-fluoro-6dEB (15F-6dEB)] fitted with a power function ($Y = aX^b$) and was determined each time samples were analyzed. Quantitation of Fet-SNPC was performed using a 1 and 2 g/L standard solution and a linear fit ($Y = aX$). All final concentrations were adjusted to compensate for water loss during fermentation.

Analysis of 15-fluoro-erythromycin concentrations was done by mass spectrometry (Sciex API100 LC< Perkin-Elmer, Wellesley, MA, USA) using roxithromycin as an internal standard [7].

Results and discussion

Effect of diketide on 6-dEB analog production

The consequence of employing the mod1Δ rather than the $KS1^0$ mutation to block the first round of condensation of polyketide production in the context of the full DEBS PKS was tested using a variety of diketide precursors. Fluoroethyl-SNPC (Fet-SNPC), vinyl-SNAC, propyl-SNPC (Pr-SNPC), and chloromethyl-SNPC (ClMe-SNPC) were fed to *S. coelicolor* CH999 employing either the $KS1^0$ or mod1Δ systems to determine the levels of utilization of each precursor and titer of the R-6dEB; 15-fluoro-, 14, 15-dihydro-, 15-methyl-, or 14-chloro-14-desmethyl-6dEB, respectively. Production of these R-6dEB, after addition of precursor at 2 mg/L, is shown in Table 2 Utilization of the different precursors varied in each strain with Fet-SNPC being utilized most efficiently in both. Fet-SNPC and vinyl-SNAC are utilized much more efficiently by the mod1Δ system than the $KS1^0$ counterpart, however both utilize Pr-SNPC with equal efficiency. In the $KS1^0$ host, vinyl-SNAC and ClMe-SNPC were utilized with equal efficiency. In the mod1Δ host, ClMe-SNPC was utilized very poorly.

In *S. coelicolor* carrying DEBS1+TE, differences between the $KS1^0$ and mod1Δ systems were observed both in the levels of production of various R-TKLs and in the utilization of various precursors [23]. However, in each case of the tested diketides, the production of R-TKL was the same or higher in those strains carrying DEBS-Mod2+TE than in those containing DEBS-$KS1^0$+TE. In contrast, in the context of the full DEBS PKS in *S. coelicolor*, the increased efficiency of the mod1Δ relative to $KS1^0$ seems to be diketide specific with improved efficiency seen with vinyl-SNAC and Fet-SNPC.

Table 2 Production of R-6dEB in *Streptomyces coelicolor* CH999 derived strains

Precursor	R-6dEB (mg/L)	
	Strain	
	JRJ2*	K279-7A*
Fluoroethyl-SNPC	622	1,220
Vinyl-SNAC	237	630
Propyl-SNPC	451	452
Chloromethyl-SNPC	234	88

R-6dEB production in *Streptomyces coelicolor* CH999 derivatives containing DEBS with either the $KS1^0$ (JRJ2*) or module 1Δ (K279-7A*) system. Two gram per Liter of the *N*-acetylcysteamine thioester (*SNAC*) or *N*-propionylcysteamine thioester (*SNPC*) intermediate was fed after 2 days growth and samples were taken at day 7 for analysis of R-6dEB production

Fluoro-diketide utilization in *Streptomyces coelicolor*

Because the highest titers were seen with Fet-SNPC, this diketide was chosen to examine utilization at varying concentrations in order to find an optimal feeding strategy. A range of concentrations from 1 to 6 g/L Fet-SNPC was tested either as a single bolus addition only on day 2 after inoculation or with additional feeds on days 3–7. Four *S. coelicolor* strains were used: JRJ2*, the CH999 host containing the $KS1^0$ system; K279-7A*, CH999 with mod1Δ system; B9, an improved strain with the $KS1^0$ system; and K460-2, the same improved strain containing the mod1Δ system. As can be seen in Table 3 , whether the background was the parent CH999 or the improved host, there was almost a twofold increase in titer and diketide utilization in the DEBS-mod1Δ system over the DEBS-$KS1^0$ system. *S. coelicolor* K279-7A* reached a maximum titer near 1.2 g/L 15F-6dEB with a 3 g/L Fet-SNPC feed. However, the best molar yield was obtained with a 1 g/L feed: 68% of the incorporated enantiomer was utilized to make 15F-6dEB. In this case, production was completed at day 5 due to the depletion of the incorporated enantiomer.

Similarly, in the host that had undergone the 6-dEB strain improvement program, utilization of the diketide and production of 15F-6dEB was also improved in the mod1Δ system (K460-2) over the $KS1^0$ system (B9). With both DEBS systems production of 15F-6dEB and SNPC utilization were superior in the improved host relative to the CH999 counterpart treated with the same amount of diketide. As can be seen in Table 3 , maximum utilization occurred at 1 or 2 g/L Fet-SNPC, although feeding higher levels of precursor increased the titer of product. In the bolus only conditions which used lower diketide feeds (2 g/L or less), the titers of 15F-6dEB ranged between 0.6 and 1.2 g/L. Production of 15F-6dEB ceased between days 3 and 4 due to depletion of the utilizable enantiomer of the precursor. Because of this complete consumption, these conditions had the highest molar yields (70–80%). With higher diketide concentrations, higher titers of 15F-6dEB (generally 2–2.5 g/L) were seen, but some of the precursor was still detectable at day 8, thus contributing to a lower molar yield.

Table 3 Comparison of 15F-6dEB and Fet-SNPC utilization between the KS1^0 and mod1Δ systems

	JRJ2*		K279-7A*		B9		K460-2	
	Titer (mg/L)	Utilization (M/M%)	Titer (mg/L)	Utilization (M/M%)	Titer (mg/L)	Utilization (M/M%)	Titer (mg/L)	Utilization (M/M%)
1 g/L	261	34	491	68	337	44	647	85
2 g/L	552	35	927	58	715	47	1,230	80
3 g/L	702	31	1,176	49	923	42	1,720	75
4 g/L	672	21	1,209	38	1,228	39	2,135	70
3 g/L+0.5 g/L/day	582	15	1,113	29	nd	nd	2,524	66
4 g/L+0.5 g/L/day	544	12	1,071	23	nd	nd	2,316	50

15F-6dEB production and diketide utilization were measured at day 8, feeding various concentrations of Fet-SNPC. Percent utilization is based on the molar ratio of the enantiomer that is incorporated in 15F-6dEB

Fluoro-diketide utilization in *Saccharopolyspora erythraea*

Diketide utilization and product formation were also examined in *S. erythraea*. The DEBS KS1^0 and mod1Δ systems were introduced into the chromosome of the erythromycin over-producing strain *S. erythraea* K41-135 to generate *S. erythraea* K39-14 [7] and K460-126A, respectively. These systems differ from their *S. coelicolor* counterparts in two ways: only a single copy of the erythromycin biosynthesis genes is present in *S. erythraea*, whereas, the DEBS genes are present on a multicopy plasmid in *S. coelicolor*; and *S. erythraea* has the capability to convert R-6dEB to its erythromycin analog. The parent strain, *S. erythraea* K41-135, has the capacity to produce up to 6.7 g/L of total erythromycin. However, as seen in Table 4 , the production of 15F-erythromycin in the KS1^0 and mod1Δ systems is very low compared to production of erythromycin in the parent strain, mostly due to the rapid degradation of the diketide by intracellular TEs leaving little available as a substrate for the DEBS PKS [7]. Peak titers obtained of 15-fluoro-erythromycin (15F-erythromycin) in the DEBS–KS1^0 system were only 33 mg/L in R5 medium when fed 2 g/L of diketide. On the other hand, there is a great improvement in diketide utilization by the strain carrying the mod1Δ system with 186 mg/L 15F-erythromycin produced. In the production medium F1-S, fed with 2 g/L of Fet-SNPC, the KS1^0 strain produced only 19 mg/L total 15F-erythromycin, while there was a tenfold increase in titer in the mod1Δ strain (220 mg/L 15F-erythromycin).

Erythromycin polyketide synthase protein levels

To examine the amount of DEBS1 protein made by the various hosts employed in the feeding experiments, *S. erythraea* K39-14 and K460-126A, as well as *S. coelicolor* B9 and K460-2, were grown in R5 media and cells were harvested at days 2 and 4. Fet-SNPC was fed to the growing cells and samples were taken at days 4 and 7 to examine production of 15F-6dEB and 15F-erythromycin. While the overall levels of 15F-6dEB or 15F-erythromycin were generally much lower in R5 than in the standard production media, the increases in diketide utilization by the mod1Δ strains were still maintained (Table 4). Total protein was normalized among the samples and a polyclonal rabbit antibody to DEBS1+TE was used as the primary antibody with an anti-rabbit-HRP secondary antibody for detection on a western blot. As can be seen in Fig. 1, there is a far stronger signal for the DEBS module 2 than KS1^0/DEBS1 protein. The same was seen in *S. erythraea* (data not shown). This increase in protein could account for the increase in substrate utilization until saturation or another downstream reaction becomes the rate-limiting step.

Table 4 15-Fluoro-6dEB productions by *Streptomyces coelicolor* and 15F-erythromycin production by *Saccharopolyspora erythraea* in R5 medium

Strain	15F-6dEB (mg/L)	15F-erythromycin (mg/L)	Diketide utilization (M/M%)
Streptomyces coelicolor B9	105		3.4
Streptomyces coelicolor K460-2	387		13
Saccharopolyspora erythraea K39-14		33	1.2
Saccharopolyspora erythraea K460-2		186	6.6

Conclusion

Precursor-directed polyketide biosynthesis has opened an avenue for development of analogues that are difficult or impossible to produce by standard chemistry

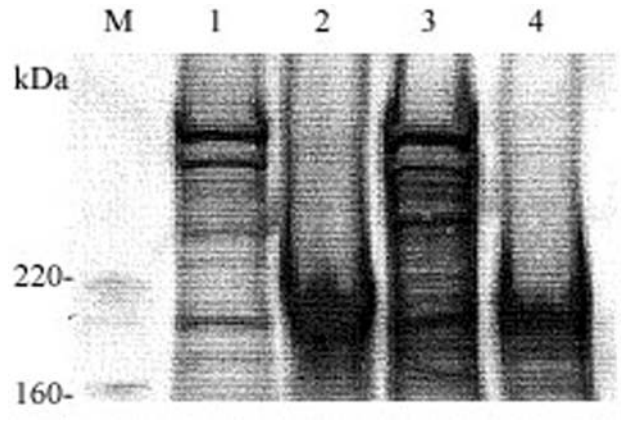

Fig. 1 Western Blot of DEBS expressed in *Streptomyces coelicolor*. Lanes 1 and 3, *Streptomyces coelicolor* JRJ2* ($KS1^0$) at days 2 and 4, respectively; Lanes 2 and 4, *Streptomyces coelicolor* K279-7A* (mod1Δ) at days 2 and 4; Lane M, molecular weight standards

approaches. However, the production of these analogues has previously been cost prohibitive due to the expense of synthesis of the thioester SNPC, degradation of the diketide in the cell, and poor utilization of the substrate by hosts with the $KS1^0$ block. Following the demonstration of increased diketide utilization and R-TKL production in *S. coelicolor* containing DEBS Mod2+TE when compared to DEBS 1/$KS1^0$+TE, strains with these same changes were developed with the entire DEBS PKS. In *S. coelicolor* CH999, the DEBS/mod1Δ system incorporated most of the examined precursors at similar or higher rates than its $KS1^0$ counterpart, although the change in utilization is diketide specific. The largest increase was observed in the utilization of Fet-SNPC to produce 15F-6dEB. Regardless of whether the genes were expressed in *S. coelicolor* CH999 or an improved host, K146-151B, there was almost a twofold increase in molar yield and productivity in the system containing the truncated DEBS1. This increase in utilization is also observed in hosts other than *S. coelicolor*. In *S. erythraea*, employing the mod1Δ rather than the $KS1^0$ system leads to an almost tenfold increase in production of 15F-erythromycin. In both *S. coelicolor* and *S. erythraea* there is a greater quantity of truncated mod1Δ-DEBS1 protein in the cell when compared with strains expressing the entire protein with the $KS1^0$ mutation. It has not yet been determined, however, if this increase is due to enhanced protein expression, increased mRNA or protein stability, or a combination of these factors. Increased protein concentration along with possibly a greater efficiency of precursor loading due to the removal of the inactive module could account for the rise in overall productivity in strains that have the loading domain and first module removed. The removal of these initial catalytic sites resulting in increased precursor utilization, together with a now simplified synthesis of the *N*-acetylcysteamine thioesters [1], has made precursor-directed biosynthesis a more economical route to the generation of novel therapeutic agents.

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